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Emergence of a novel recombinant porcine circovirus type 2b (PCV2b) and PCV2d in Thailand

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Abstract

Porcine circovirus type 2 (PCV2) has been recognized as a causative agent of porcine circovirus-associated diseases (PCVAD) affecting the global swine industry for more than three decades. The high mutation rate of PCV2 can lead to rapid evolution and emergence of novel PCV2 strains. In this study, we investigated the genetic diversity of PCV2 strains circulating in Thailand between 2019-2020. From 734 samples (145 farms), 396 (54.0%) samples obtained from 118 farms were positive for PCV2. Of these, whole-genome sequences of 51 Thai PCV2 strains were analyzed and assigned to PCV2d (86.3%) and PCV2b (13.7%). The complete genomic analysis also revealed an intergenotypic recombinant virus between PCV2b and PCV2d. Our findings and viral sequence dataset provide essential information on the genetic diversity of PCV2, including the evidence of novel intergenotypic recombination of PCV2 in Thailand.

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Abstract

Porcine circovirus type 2 (PCV2) has been recognized as a causative agent of porcine circovirus-associated diseases (PCVAD) affecting the global swine industry for more than three decades. The high mutation rate of PCV2 can lead to rapid evolution and emergence of novel PCV2 strains. In this study, we investigated the genetic diversity of PCV2 strains circulating in Thailand between 2019-2020. From 734 samples (145 farms), 396 (54.0%) samples obtained from 118 farms were positive for PCV2. Of these, whole-genome sequences of 51 Thai PCV2 strains were analyzed and assigned to PCV2d (86.3%) and PCV2b (13.7%). The complete genomic analysis also revealed an intergenotypic recombinant virus between PCV2b and PCV2d. Our findings and viral sequence dataset provide essential information on the genetic diversity of PCV2, including the evidence of novel intergenotypic recombination of PCV2 in Thailand.

KEYWORDS

Porcine circovirus type 2, genetic diversity, swine, recombination, Thailand

INTRODUCTION

The exports of live pigs and pork products from Thailand have increased since the second half of 2019 due to African swine fever (ASF) outbreaks in China and the neighboring countries in Southeast Asia (SEA). The role of international trade and globalization facilitates the potential spread of many transboundary animal diseases, which later become endemic in the areas. Porcine circovirus type 2 (PCV2) emerged a few decades ago and has caused economic losses to the swine industry globally. The virus induces numerous syndromes and diseases called porcine circovirus-associated disease (PCVAD) consisting of postweaning multisystemic wasting syndrome (PMWS), multisystemic inflammation, porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, and respiratory disease (Opriessnig & Langohr, 2013; Segales, 2012). The disease manifestations caused by PCV2 vary widely in their severity, ranging from none to severe clinical signs affecting directly or indirectly the growth performance.

PCV2 is a non-enveloped single-stranded DNA virus containing a circular genome of 1766-1768 nucleotides (nt) (L. J. Guo, Lu, Wei, Huang, & Liu, 2010) containing three main open reading frames (ORFs). Replicase protein encoded by ORF1 (*Rep* gene) is essential for viral replication (Mankertz, Buhk, Blaess, & Mankertz, 1998). Capsid protein encoded by ORF2 (*Cap* gene) is a viral structural protein playing a significant role in the immunogenicity, virulence, and characteristics of the virus genotypes (Nawagitgul et al., 2000; Olvera, Cortey, & Segales, 2007). Additionally, a non-structural protein encoded by ORF3 could induce apoptosis (Olvera et al., 2007).

PCV2 rapidly evolves prompting, consequently, the emergence of new virus genotypes. To date, the virus is classified into eight distinct genotypes (PCV2a-2h) based on the nucleotide sequence of capsid gene (Franzo & Segales, 2018). The virus shows a high mutation rate (i.e., 10^{-3}-10^{-4} substitution/site/year) (Franzo, Cortey, Segales, Hughes, & Drigo, 2016) and phylodynamic studies revealed two major genetic shifts: PCV2a towards PCV2b around 2003 and PCV2b towards PCV2d around 2012 (Wang et al., 2020). Recently, PCV2d has overtaken PCV2b and become the predominant genotype worldwide (Xiao, Halbur, & Opriessnig, 2015). PCV2d might derive from the solid selection pressure or vaccine-driven selection pressure by the massive vaccination worldwide and the virus might evolve to enhance its virulence compared with the previously existing PCV2a and PCV2b (L. Guo et al., 2012). Beside the point mutation, recombinant PCV2 strains has also been reported in some countries such as China, India, and South Korea (Jang, Yoo, Kim, Yang, & Lee, 2021; S et al., 2021; Wei et al., 2019). Hence, intra-genotypic or inter-genotypic recombination between the predominant PCV2d and the coexisting genotypes is possible.

In Thailand, the complete genetic data for each PCV2 genotype are limited and have not been updated since 2015 (Thangthamniyom et al., 2017). Therefore, this study aimed to investigate the current prevalence and genetic diversity of the Thai PCV2 based on the whole-genome sequences. The findings may represent the genetic variation of PCV2 not only in Thailand but also in parts of the SEA region. In addition, this

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study found a novel recombinant strain originating from PCV2b and PCV2d with recombination breakpoints located in the *Rep* and *Cap*genes (ORF1 and ORF2 regions). Thus, the information obtained from this study will be useful for better understand the current molecular epidemiology and genetic recombination of PCV2.

2. MATERIALS AND METHODS

2.1 Sample collection and processing

A total of 734 samples from various submitted sample types, including serum, pooled tissue samples, semen, feces, aborted fetuses, and oral fluids, were obtained from Chulalongkorn University, Veterinary Diagnostic Laboratory (CU-VDL) and Diagnostic Laboratory of Large Animal Hospital and Students Training Center between January 2019 and December 2020. The samples were collected from 145 swine farms located in 18 provinces from different geographical regions of Thailand, mainly in the high pig density areas in the Western, Central, and Eastern parts (Figure S1). The experimental protocol was approved by the certification of the institutional biosafety committee of the Faculty of Veterinary Science, Chulalongkorn University (protocol number 2131007).

2.2 Viral DNA extraction

Viral DNA was extracted by using the IndiMag Pathogen kit of viral RNA/DNA (Indical Bioscience, Germany) on the automated extraction platform according to the manufacturer's instruction. The obtained DNA was stored at -80°C until used.

2.3 PCV2 detection with real-time PCR assay

PCV2 real-time PCR screening was performed as previously described (Wang et al., 2019). Briefly, PCR reactions were performed in a 20-ul reaction containing 4 ul of extracted DNA, 0.2 uM each of forward and reverse primers, 0.1 uM each of probes, and 10 ul of Luna® Universal Probe qPCR master mix (NEB, MA, USA). PCV2 was detected using Quantstudio5 real-time system (Appiled Biosystems, USA). The PCR condition consisted of initial denaturation at 94°C for 30s followed by 45 cycles of 94°C for 15s, and 60°C for 45s.

2.4 PCV2 Whole-genome sequencing

For PCV2 characterization, the PCV2 PCR positive samples with a ct < 30 were selected for whole-genome sequencing. The samples were further selected to cover different profiles of the samples, including the date of sample collection and the geographical regions of the farms. PCV2 whole-genome samples were amplified using two pairs of overlapping primers as previously described (An, Roh, Song, Park, & Park, 2007; Fenaux, Halbur, Gill, Toth, & Meng, 2000) (Table S1). All PCR reactions were performed in 50 ul reaction mixtures containing 3 ul of the extracted DNA, 0.2 uM of each forward and reverse primers (PCV2-sF1/PCV2_sR1 and PCV2-sF2/PCV2-sR2), and 25 ul of Onetaq® 2x Master Mix (NEB, MA, USA). The PCR condition consisted of initial denaturation at 94°C for 30s, followed by 35 cycles of 94°C for 30s, 55°C for 45s, 68°C for 90s, and 68°C for 5min. PCR products were visualized by 1% Agarose gels containing nucleic acid staining. Successfully amplified samples were purified using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL, Germany) and submitted for sequencing by barcode-tagged sequencing platform (Celemic, Seoul, Korea). The nucleotide sequences were further assembled and validated with SeqMan and EditSeq software v.5.03 (DNASTAR Inc., Madison, Wisconsin, USA) and submitted to GenBank.

2.5 Phylogenetic analysis

For genetic analysis of PCV2, nucleotide sequences and deduced amino acids from this study were aligned with different reference strains and sequences from adjacent countries, which were retrieved from the GenBank database. The alignments were conducted using the Clustal W algorithm of BioEdit 7.2.5 (https://bioedit.software.informer.com/). For phylogenetic analysis, the ORF1 and ORF2 genes were aligned with reference strains of different PCV2 genotypes, and the phylogenetic tree was constructed with MEGA version 10.2.6 using neighbor-joining algorithm and bootstrap analysis of 1000 replications (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

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2.6 Recombination analysis

The whole-genome sequences were aligned and analyzed with a recombination detection program (RDP version 4.22) (Martin et al., 2010) to investigate recombinant events. The general setting algorithm supported the recombination breakpoint detection using seven recombination detection methods, namely RDP, GENECOV, Bootscan, MaxChi, Chimaera, SiScan, and 3Seq, respectively. Only recombination events identified by at least five methods with the p -values cut-off of 0.01 were accepted. Bonferroni correction was applied throughout the analysis. Furthermore, the aligned sequences were re-analyzed with SIMPLOT software v. 3.5 by Bootscan methods to confirm the potential recombination sequences (Lole et al., 1999). Phylogenetic tree analysis was reconstructed for putative recombinant data of separate regions of the proposed parental strains.

3. RESULTS

3.1 Prevalence and genomic characterization of PCV2

Seven hundred and thirty-four samples from 145 swine farms from different geographical regions were submitted to CU-VDL during 2019-2020. Real-time PCR was conducted on all samples for PCV2 detection. The results are shown in Table 1 and Figure S1.

3.2 Phylogenetic analysis of PCV2

In this study, fifty-one PCV2 isolates were selected from 49 farms during 2019-2020 for genetic characterization based on the previously described criteria. The nucleotide sequences of PCV2 were deposited in the NCBI GenBank database under accession no. OL677572-OL677622 (Table 2).

Genetic analysis using complete genome sequences showed that all 51 PCV2 isolates during 2019-2020 were 1,767 nt in length containing 945 nt of ORF1 and 702-705 nt of ORF2. Phylogenetic analysis based on ORF2 and genome sequences revealed that 86.27% (44 out 51) of the strains belong to PCV2d and the remaining 13.73% (7 out 51) were PCV2b (Figure 1). Notably, PCV2b and PCV2d could be found from the same farms for two farms (farm A003 and A036, Table 2).

Phylogenetic trees based on the genome, ORF1 and ORF2 showed consistent topology except for one isolate, 19NPT29. Based on the genome and ORF2 sequences, 19NPT29 was in the PCV2d cluster; however, it was in the PCV2b cluster based on the ORF1 sequences (Figure 1).

Further investigation of deduced amino acid sequences of ORF2 was performed by pairwise alignment with reference strains from various genotypes. The ORF2 amino acid variations were analyzed focused on antibody recognition sites from four regions at positions 51-84, 113-139, 161-207, and 228-233 (Lekcharoensuk et al., 2004; Mahe et al., 2000). All the seven PCV2b isolates in this study had typical SNPRSV and A/TGIE motifs without unique amino acid substitution. Likewise, 43 of 44 PCV2d isolates have typical SNPLTV and TGID motifs corresponding to PCV2d strains. Notably, 19NPT29 isolate presented the SNPRSV and TGID motifs, belonging to the PCV2b and PCV2d, respectively, suggesting the recombination event. In this study, 31of 44 PCV2d isolates exhibited unique amino acids at four positions: H133, D134, M136, and K232 (Figure S2). Some of these variable regions correspond to antibody recognition sites at positions 51-84, 113-139, 161-207, and 228-233 (Lekcharoensuk et al., 2004; Mahe et al., 2000).

3.3 Recombination analysis

Previously, recombination has been observed in various PCV2 strains (Cai et al., 2011; Jang et al., 2021; Kleymann et al., 2020; Neira et al., 2017; Ramos, Mirazo, Castro, & Arbiza, 2013; Wei et al., 2019). This study determined genetic recombination analysis using seven methods (RDP, GeneConv, BootScan, Maxchi, Chimera, SiScan, and 3Seq) implemented in RDP software. The results showed that 19NPT29 was identified as an intergenotypic PCV2b/PCV2d recombinant by all seven methods with a high degree of statistical support (average p -value = 3.84 x 10^{-9}) (Table 3). The recombination analysis also revealed PCV2b (South Korea/2016/KU-1605) and PCV2d (Thailand/2019/19RBR10) as the major and minor parents, respectively. Furthermore, the putative recombination breakpoints were determined in *Rep* and *Cap* genes at

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nucleotide positions 508 and 1356. The recombination was also confirmed using SIMPLOT software (data not shown). For further analysis, the 19NPT29 genome was divided into two regions, 'recombinant region 1' and 'recombinant region 2', using the recombination breakpoints (Figure 2).

The recombination events of 19NPT29 were also achieved by using nucleotide-by-nucleotide comparisons with the major and minor parental strains, as shown in Figure 2. In recombinant region 1, 19NPT29 showed a higher nucleotide identity of 99.8% with 19RBR10, while it showed 95.7% nucleotide identity with KU-1605 (Table 4). On the other hand, in recombinant region 2, 19NPT29 showed a higher nucleotide identity of 99.9% with KU-1605 and showed 95% nucleotide identity with 19RBR10. Furthermore, the recombination event in 19NPT29 was confirmed by PCR amplification and analysis of a nucleotide fragment (nt 453-1598) covering the recombination breakpoint (data not shown).

Taken together, it is the first report in Thailand revealing a novel emerging PCV2 variant generated by intergenotypic recombination between PCV2b and PCV2d.

4. DISCUSSION

Porcine circovirus type 2 is one of the major swine viruses causing enormous economic losses to the swine industry globally. Although commercially available vaccines have been widely used in the last two decades, vaccine failures remain questionable. PCV2 has a high nucleotide substitution rate that varies between $10^{-3}$and 10^{-4} substitution/site/year, similar to RNA viruses (Franzo et al., 2016). In addition, both point mutation and recombination contribute to the evolution of PCV2 (Firth, Charleston, Duffy, Shapiro, & Holmes, 2009), which could facilitate the evasion of the host immune responses. To date, at least eight PCV2 genotypes (A to H) were classified based on the phylogenetic analysis (Franzo & Segales, 2018).

In Thailand, data on the prevalence and genetic diversity of PCV2 in the past five years are limited. In this study, the prevalence and genetic diversity of PCV2 in Thailand were investigated, and the high prevalence of PCV2 (53.95% (396/734)) was observed in Thai pig populations from wide geographical regions during 2019-2020. In the current study, PCV2d was a dominant genotype, which is in agreement with the previous report (Thangthamniyom et al., 2017). Not only that, the proportion of PCV2d in Thailand seems to be increasing, indicating that the predominant PCV2d was gradually taking over other genotypes in the country. During 2009-2015, 54.81% of Thai PCV2 were PCV2d, while 40% were PCV2b (Thangthamniyom et al., 2017). The proportion of PCV2d in this study was increased to 86.27% in 2019-2020, and PCV2b was dropped to 13.73%. This increasing number of PCV2d infections could raise awareness regarding the PCV2 vaccine efficacy, mainly derived from PCV2a or PCV2b.

Other than point mutation, natural recombination can also lead to the emergence of novel PCV2 variant strains. Various studies demonstrated that both intra- and inter-genotypic recombination were found in many PCV2 strains (Cai et al., 2011; Cheung, 2009; Hesse, Kerrigan, & Rowland, 2008; Huang et al., 2013; Jang et al., 2021; Kim et al., 2009; Kleymann et al., 2020; Ma et al., 2007; Ramos et al., 2013). However, the evidence for genetic recombination of PCV2 is scant in Thailand due to the lack of complete genome sequences datasets. In this study, the presence of both PCV2d and PCV2b in the same farm (but at different time points) was occasionally found. Coincidental infection with different PCV2 genotypes might provide an opportunity for natural inter-genotypic recombination.

One recombinant PCV2 strain was identified in this study, 19NPT29. The results showed that the recombinant virus might be generated from a 19RBR10-like strain (PCV2d) and a KU-1605-like strain (PCV2b). Evidently, all Thai PCV2b isolates shared 99.6-99.7% nucleotide sequence identity with the South Korean KU-1605 (data not shown), which was suspected to be a parental strain of most Thai PCV2b isolates. Therefore, it is possible that PCV2b from South Korea (e.g., KU-1605) might be introduced into Thailand via non-pig transmission sources before spreading in the Thai pig populations and then being recombined with the local Thai PCV2d strains (e.g., 19RBR10).

The time and place where the recombination events occurred in 19NPT29 are unknown, mainly due to the limited number of samples from the farm of origin and the short duration of the study. The virus was found

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in a pig farm in Nakornpathom in 2019. During the study, there was only one sample submission from this farm, in which 19NPT29 was identified. Co-circulation of PCV2b and PCV2d in this farm was not observed. In fact, 19NPT29 was the only PCV2 strain found in this farm. It is possible that co-circulation of PCV2b and PCV2d took place in this farm prior to the investigation and gave rise to the emergence of 19NPT29. Alternatively, 19NPT29 might emerge in the other area and then spread to this farm.

Recombination breakpoints can be found in both ORF1 and ORF2 of recombinant PCV2 strains. ORF1 was predicted to be a primary target for inter-genotypic recombination (Hesse et al., 2008; Kim et al., 2009), possibly from the high conservation of nucleotide sequences in this region between the two genotypes that facilitating the recombination events. On the other hand, ORF2 encoded for the capsid gene that plays a significant role in immunogenicity, the virulence of the virus, and grouping the virus genotypes (Nawagitgul et al., 2000; Olvera et al., 2007). Hence, recombination events within ORF2 may contribute to both antigenicity and virulence of the novel virus (Franzo et al., 2016). Two recombination breakpoints were identified in ORF1 and ORF2 genes at positions 508 and 1356 of 19NPT29. Therefore, the antigenicity and pathogenesis of this recombinant virus should be further studied. Although the virus was detected in pigs that showed respiratory signs, the clinical outcomes can be affected by many factors.

For deduced amino acid analysis, the ORF2 gene encoded for capsid protein is recognized for phylogenetic analysis and still plays a vital role in both antigenic and immunogenic properties. Besides, antibody recognition sites were described from four regions at positions A (51-84), B (113-139), C (161-207), and D (228-233) in the Capsid protein (Lekcharoensuk et al., 2004; Mahe et al., 2000). In the present study, 31 deduced PCV2d ORF2 amino acid sequences presented four unique amino acid mutations corresponding to antibody recognition regions compared to different PCV2 genotypes. Specific mutations of PCV2d mainly occurred at positions 133, 134, 136 and 232 located in immunoreactive domains B and D. Notably, mutational events within the critical epitopes, especially at amino acid 133-135, may be responsible for the virulence of PCV2 (Krakowka et al., 2012). Hence, the mutations in the epitope regions of Thai PCV2d isolates may enhance the virus's ability to escape the host immune responses and contribute to the disease severity. The effects of amino acid variation found in this study need further investigation to clarify the pathogenicity and virus properties.

To date, both intra-genotypic and inter-genotypic recombination of PCV2 have been reported worldwide, including Chile (Neira et al., 2017), China (Cai et al., 2011; Huang et al., 2013; Ma et al., 2007), Uruguay (Ramos et al., 2013), USA (Cheung, 2009; Hesse et al., 2008), South Korea (Jang et al., 2021; Kim et al., 2009), the Lesser Antilles in the Caribbean Sea (Kleymann et al., 2020), and lastly, Thailand. Therefore, recombination analysis should not be neglected in PCV2 genetic studies (e.g., virus evolution, phylogenetic analysis).

In conclusion, this study reveals PCV2d as a dominant PCV2 genotype in Thailand and an emergence of a recombinant virus between PCV2b and PCV2d through inter-genotypic recombination. Regular surveillance and monitoring in the farms may help gain a comprehensive view of PCV2 genetic evolution to implement early interventions against the emergence of PCV2 variants. Further investigation is needed to increase our understanding of the pathogenicity and virulence of the recombinant virus.

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CONFILCT OF INTEREST

The authors declare no conflicts of interests regarding the present research.

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Table 1 The prevalence of PCV2 in all tested samples during 2019-2020

		Types of sample	Types of sample	Types of sample	Types of sample	Type
Periods	Group of pigs+	Internal organs	Serum	Fetus	Feces	Sem
Jan-Apr 2019	Sucking	4/5	-	-	4/4	-
	Nursery	24/29	11/31	-	-	-
	Fattening	16/16	2/12	-	-	-
	Breeder	2/2	11/31	15/17	-	0/6
May-Aug 2019	Sucking	4/5	0/7	-	0/3	-
	Nursery	30/34	10/61	-	0/1	-
	Fattening	9/9	1/9	-	0/1	-
	Breeder	0/2	3/15	6/13	1/1	1/4
Sep-Dec 2019	Sucking	2/3	-	1/1	-	-
	Nursery	12/12	3/6	-	-	-
	Fattening	3/3	12/17	-	-	-
	Breeder	-	0/2	1/3		-
Jan-Apr 2020	Sucking	0/2	0/2	-	-	-
	Nursery	17/17	7/10	-	0/2	-
	Fattening	-	3/3	-	-	-
	Breeder	-	1/8	8/10	-	1/4
May-Aug 2020	Sucking	3/6	2/2	-	0/1	-
	Nursery	13/13	9/19	-	1/1	-
	Fattening	12/13	10/12	-	-	-
	Breeder	0/1	5/9	9/18	-	0/4
Sep-Dec 2020	Sucking	-	0/9	-	-	-
	Nursery	8/13	7/24	-	0/1	-
	Fattening	2/2	77/117	-	-	-
	Breeder	0/1	4/21	5/14	-	0/4
Jan 2019-Dec 2020	Jan 2019-Dec 2020	161/188 (85.6%)	178/427 (41.7%)	45/76 (59.2%)	6/15 (40%)	2/22

+ Sucking: <4 weeks; Nursery: 5-8 weeks; Fattening: 9-20 weeks; Breeder: boar, gilt, and sow

++ The PCV2 positive farms were calculated during Jan2019-Dec2020.

Table 2 Detail description of Thai PCV2 isolates characterized in this study

No	Virus	Year	Farm	Group of pigs	Clinical signs	Province	Genotype	Accession
1	19CBI2	2019	A025	Fattening	NA	Chonburi	PCV2d	OL677573
2	19CBI262	2019	A004	Fattening	Respiratory sign	Chonburi	PCV2d	OL677574
3	19CMI162	2019	A001	Fattening	NA	Chiang Mai	PCV2d	OL677575
4	19NPT1	2019	A029	Nursery	Respiratory sign	Nakornpathom	PCV2d	OL677576
5	19NPT269	2019	A009	Nursery	Respiratory sign	Nakornpathom	PCV2d	OL677577
6	19NPT282	2019	A010	Fattening	Respiratory sign	Nakornpathom	PCV2d	OL677578
7	19NPT29	2019	A028	Fattening	Respiratory sign	Nakornpathom	mPCV2	OL677579

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No	Virus	Year	Farm	Group of pigs	Clinical signs	Province	Genotype	Accession
8	19NPT3	2019	A027	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67758
9	19NPT35	2019	A030	Nursery	NA	Nakornpathom	PCV2b	OL67758
10	19NPT4	2019	A015	Fattening	Sudden death	Nakornpathom	PCV2d	OL67758
11	19PBI235	2019	A022	Nursery	NA	Phetchaburi	PCV2b	OL67758
12	19PRI56	2019	A021	Nursery	Respiratory sign	Prachinburi	PCV2d	OL67758
13	19RBR10	2019	A014	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67758
14	19RBR150	2019	A023	Nursery	NA	Ratchaburi	PCV2d	OL67758
15	19RBR20	2019	A012	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67758
16	19RBR217	2019	A018	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67758
17	19RBR230	2019	A003	Fattening	Respiratory sign	Ratchaburi	PCV2b	OL67758
18	19RBR24	2019	A016	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67759
19	19RBR242	2019	A002	Nursery	Respiratory sign	Ratchaburi	PCV2b	OL67759
20	19RBR247	2019	A003	Fattening	Respiratory sign	Ratchaburi	PCV2d	OL67759
21	19RBR25	2019	A017	Nursery	Respiratory sign, seizure	Ratchaburi	PCV2d	OL67759
22	19RBR263	2019	A007	fetus	Abortion	Ratchaburi	PCV2d	OL67759
23	19RBR264	2019	A006	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67759
24	19RBR281	2019	A013	Fattening	Respiratory sign	Ratchaburi	PCV2d	OL67759
25	19RBR365	2019	A005	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67759
26	19RBR374	2019	A008	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67759
27	19RBR47	2019	A024	Nursery	Respiratory sign	Ratchaburi	PCV2b	OL67759
28	19RBR58	2019	A020	Sucking pig	NA	Ratchaburi	PCV2d	OL67760
29	19RBR6	2019	A026	Nursery	Respiratory sign	Ratchaburi	PCV2b	OL67760
30	19RBR60	2019	A019	Fattening	Diarrhea	Ratchaburi	PCV2d	OL67760
31	19RBR8	2019	A011	Nursery	Respiratory sign	Ratchaburi	PCV2d	OL67760
32	20CBI363	2020	A046	Nursery	Respiratory sign	Chonburi	PCV2d	OL67760
33	20CBI61	2020	A037	Fattening	Respiratory sign, lameness	Chonburi	PCV2d	OL67760
34	20CBI97	2020	A033	Nursery	NA	Chonburi	PCV2d	OL67760
35	20KKN424	2020	A045	Fattening	Stunt growth	Khonkaen	PCV2d	OL67760
36	20NPT29	2020	A032	Nursery	Respiratory sign	Nakornpathom	PCV2d	OL67760
37	20NPT366	2020	A041	Nursery	NA	Nakornpathom	PCV2d	OL67760
38	20NPT53	2020	A035	Fattening	NA	Nakornpathom	PCV2d	OL67761
39	20NPT90	2020	A031	Nursery	Respiratory sign, stunt growth	Nakornpathom	PCV2d	OL67761
40	20PBI396	2020	A044	Nursery	Respiratory sign	Phetchaburi	PCV2d	OL67761
41	20RBR256	2020	A039	Fattening	Respiratory sign	Ratchaburi	PCV2d	OL67761
42	20RBR357	2020	A040	Fattening	NA	Ratchaburi	PCV2d	OL67761
43	20RBR377	2020	A042	Nursery	Respiratory sign	Ratchaburi	PCV2b	OL67761
44	20RBR395	2020	A002	Nursery	Stunt growth	Ratchaburi	PCV2d	OL67761
45	20RBR85	2020	A038	Nursery	NA	Ratchaburi	PCV2d	OL67761
46	20RBR91	2020	A034	Nursery	NA	Ratchaburi	PCV2d	OL67761
47	20SPB55	2020	A036	Fattening	Respiratory sign	Suphanburi	PCV2d	OL67761
48	20SPB72	2020	A036	Fattening	Respiratory sign	Suphanburi	PCV2d	OL67762
49	20TRG444	2020	A043	Fattening	Stunt growth, pale	Trang	PCV2d	OL67762
50	20UDT433	2020	A047	Fattening	NA	Udonthani	PCV2d	OL67762
51	20UTI438	2020	A048	Fattening	NA	Uthaithani	PCV2d	OL67762

Table 3 Genetic recombination identification methods using RDP4 software.

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Recombinant	Major parent	Minor parent	Beginning breakpoint	Ending breakpoint	Detection methods	Detection methods	Detection methods	Detection methods	Detection methods	Detection methods
					R	**G**	**B**	**M**	**C**	**S**
Thailand 19NPT29 (PCV2b-PCV2d)	South Korea KU-1605 (PCV2b)	Thailand 19RBR10 (PCV2d)	508	1356	+	+	+	+	+	+

*** R= RDP, G= GENECONV, B= Bootscan, M= Maxchi, C=Chimaera, S= SiScan, T=3Seq

Table 4 Nucleotide sequence comparison at genetic marker positions between recombinant PCV2 and its parental strains

strains	Nucleotide positions	Nucleotide positions	Nucleotide positions	Nucleotide positions	Nucleo
	551	587	716	737	752
KU-1605	T	T	C	G	A
19RBR10	G	C	G	C	T
19NPT29	G	C	G	C	T
	1207	1222	1225	1228	1229
KU-1605	T	A	A	G	G
19RBR10	G	G	T	C	C
19NPT29	G	G	T	C	C

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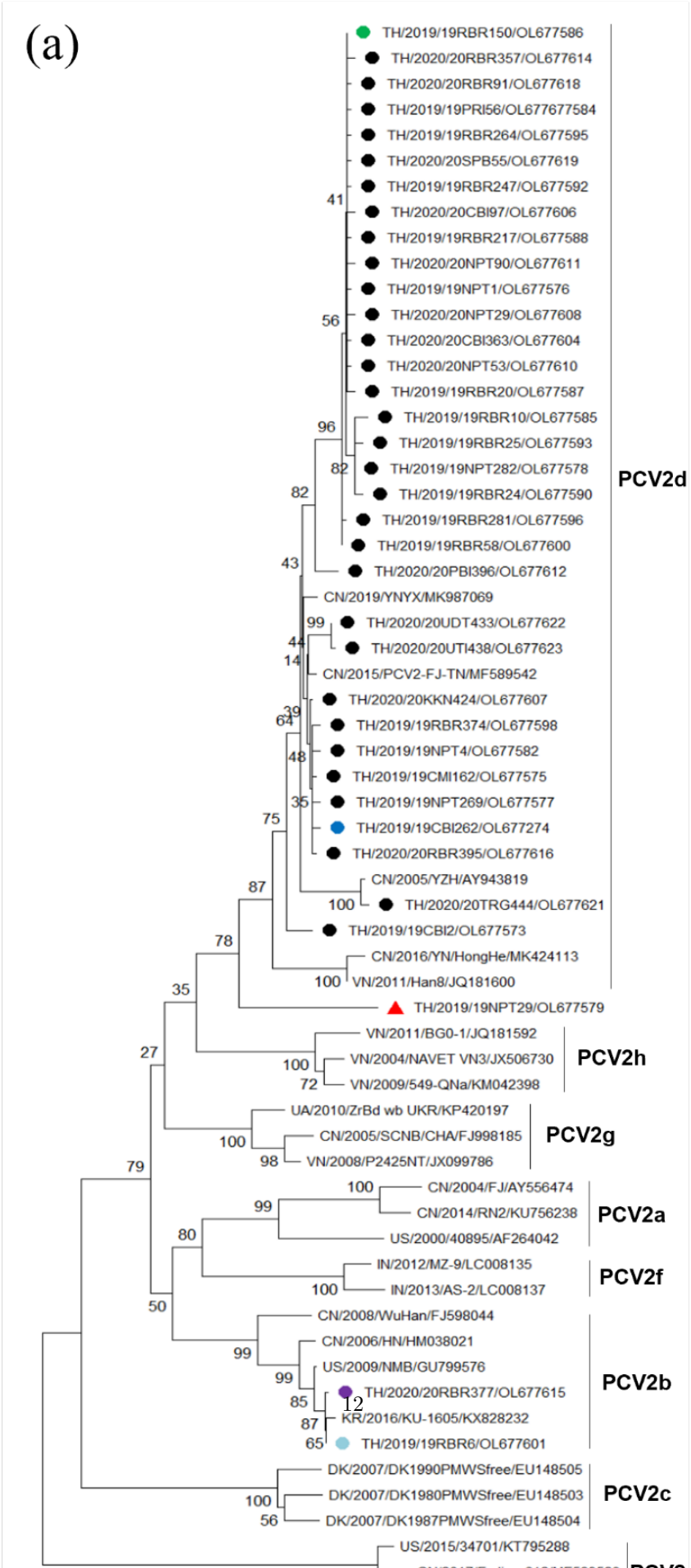

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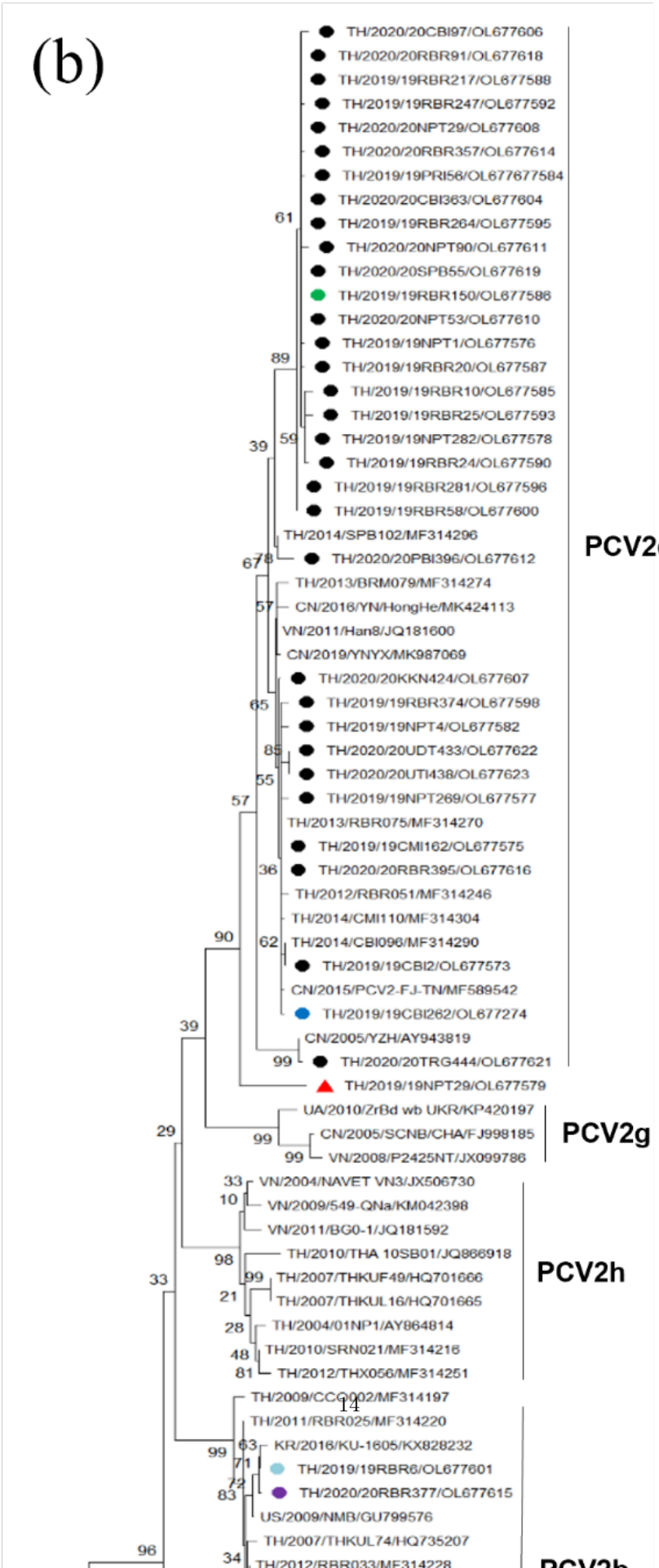

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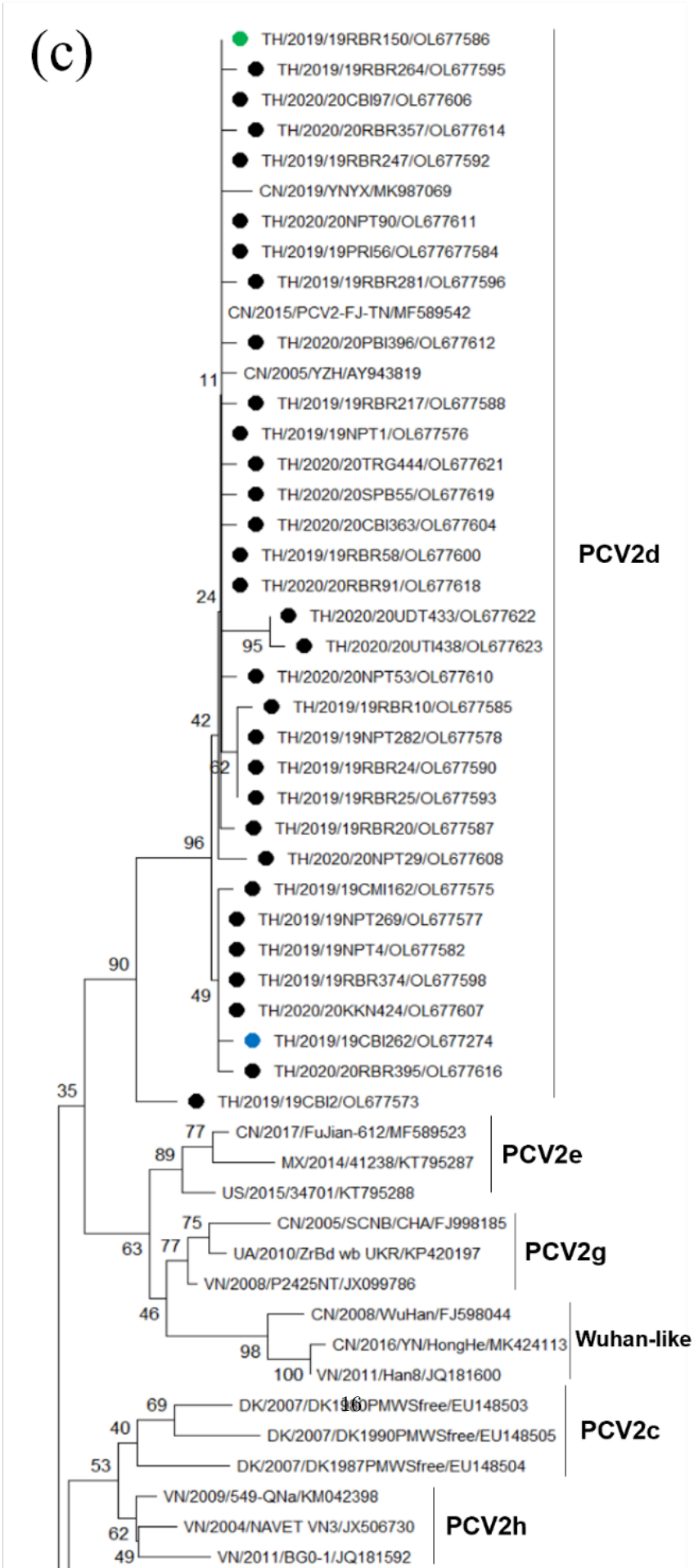

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Figure 1. Phylogenetic tree based on the complete genome sequences (a), *Cap* gene (ORF2) (b), and *Rep* gene (ORF1) (c) using the neighbor-joining method with a p-distance model and bootstrapping at 1000 replicates. The Thai PCV2 sequences obtained in this study were marked with solid black and colored circles. The solid-colored circles indicate the representative sequences of PCV2 isolates that showed 100% similar identity in each group (Table S2). A red triangle indicates the recombinant PCV2 strain.

Figure 2. Recombination analysis generated by RDP software and genetic organization of recombinant (19NPT29). Bootscanning analysis was performed with 19NPT29 as query sequence using a sliding window of 200 nt moving in 20 nt steps. A purple shaded area of 19NPT29 indicates the recombination region at positions 508-1356. The phylogenetic trees were constructed based on recombinant region 1 (purple shaded) and recombinant region 2 (dark green shaded) to confirm recombination event.